

**Response to Restriction Requirement
Communication Regarding Entry of Sequence Listing, and
Preliminary Amendment**

Page 2

Applicant(s): Tarleton et al.

Serial No.: 09/518,156

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For: PROPHYLACTIC AND THERAPEUTIC IMMUNIZATION AGAINST PROTOZOAN AND DISEASE

22
secreted polypeptide. Surface associated-immunogenic polypeptides include, for example, *T. cruzi* proteins that are anchored to the plasma membrane by glycosylphosphatidylinositols, or GPIs, and those that have transmembrane domains or are otherwise embedded in the plasma membrane. One class of polypeptides that exemplifies immunogenic polypeptides according to the invention is the trans-sialidase family of proteins, such as TSA-1 (*T. cruzi* Peru; D. Fouts et al., Mol. Biochem. Parasitol. 46:189-200 (1991); GenBank Acc. Number M58466), ASP-1 (*T. cruzi* Brazil; M. Santos et al., Mol. Biochem. Parasitol. 86:1-11 (1997); GenBank Acc. Number U74494) and ASP-2 (*T. cruzi* Brazil; H. Low et al., Mol. Biochem. Parasitol. 88:137-149 (1997); GenBank Acc. Number U77951), which are found in both secreted and surface-displayed forms; other examples are proteins that are secreted upon entry of the host cells by *T. cruzi*, such as the hemolysin, and the Lyt1 protein (porin).

Please replace the paragraph beginning at page 45, line 1 through line 14, with the following rewritten paragraph. Pursuant to 37 C.F.R. §1.121, this paragraph is also shown in Appendix A with notations to indicate the changes made.

45
Peptides. Peptides were synthesized using Fmoc-based solid phase chemistry on an ACT MPS 350 peptide synthesizer (Advanced Chem Tech, Louisville, KY) by the Molecular and Genetic Instrumentation Facility (MGIF) at the University of Georgia. The synthetic peptides pep77.2 (TSA-1515-522: VDYNFTIV (SEQ ID NO:1)) (B. Wizen et al., J. Immunol. 159:6120-30 (1997); M. Santos et al., Mol. Biochem. Parasitol. 86:1-11 (1997)), PA8 (ASP-2552-559: VNHRFTLV(SEQ ID NO:7)) and PA14 (ASP-1509-516: VNHDFTVV (SEQ ID NO:8)) (H. Low et al., J. Immunol. 160:1817-1823 (1998)) represent H-2K^b restricted CTL epitopes from *T. cruzi* proteins TSA-1, ASP-2 and ASP-1, respectively. The H-2K^b-restricted chicken ovalbumin CTL epitope OVA257-264 (SIINFEKL; SEQ ID NO:2) was used as a control peptide (O. Rotzschke et al., Eur. J. Immunol. 21:2891-4 (1991)). Lyophilized peptides were dissolved at 5 mM concentration in sterile phosphate buffer saline (PBS, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, 137 mM NaCl, 8 mM KCl, pH 7.4) and stored at -20°C.

**Response to Restriction Requirement
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Page 3

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Please replace the paragraph beginning at page 68, line 17 through line 29, with the following rewritten paragraph. Pursuant to 37 C.F.R. §1.121, this paragraph is also shown in Appendix A with notations to indicate the changes made.

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Generation of a T. cruzi ORF-library in a eukaryotic expression vector. The ORF.GFP library clones from each 96-well plate are pooled together. Plasmid DNA isolated from the plate pools is digested with *PacI* and *AscI* restriction enzymes, separated on 1% agarose gels. *T. cruzi* genomic DNA fragments (0.2 to 1kB) are purified and ligated in CMV.UB.PBA vector digested with similar enzymes. The CMV.UB.PBA vector is designed such that the *T. cruzi* genomic DNA inserts are transcribed through strong cytomegalovirus promoter and the translation initiates with a ubiquitin terminal fusion. This vector when used for genetic immunization allows high level production of the fusion protein, and the antigenic protein is targeted to the proteosome for efficient processing and presentation. The *T. cruzi* genomic library in eukaryotic expression vector CMV.UB.PBA is then screened using the ELI approach for the isolation of individual protective genes.

Please replace the paragraph beginning at page 76, line 14, through page 77, line 4, with the following rewritten paragraph. Pursuant to 37 C.F.R. §1.121, this paragraph is also shown in Appendix A with notations to indicate the changes made.

15
(iii) Detection of *T. cruzi* by in situ PCR: In situ PCR amplification of *T. cruzi* minicircle kinetoplast DNA (kDNA) has been used to detect parasites or parasite derived DNA in infected tissue. The high sensitivity of this procedure is enhanced by the fact that each parasite has 5-10,000 copies of minicircles, and each minicircle has 4 copies of conserved regions, which are to be used as targets for PCR amplification of kDNA. If parasites can not be detected in immunized mice by enzymatic and competitive PCR techniques, then further confirmation of clearance of parasites and cure from disease will be done by monitoring the presence of parasites in tissues of the mice by in situ PCR. Tissues from the heart or skeletal muscle of infected mice will be processed, sectioned and fixed as previously described (60). kDNA-specific oligonucleotides 5'-GGTTCGATTGGGGTTGGTGTAAATATA-3' (SEQ ID

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Page 4

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NO:16) and biotin-labeled 5'-AAATAATGTACGGGT/GGAGATGCATGA-3' (SEQ ID NO:17) as forward and reverse primers will be used for amplification by PCR. PCR products will be detected with avidin-peroxidase and color developed with 3',3'-diaminobenzidine tetrahydrochloride. Sections will be counter-stained with hematoxylin and visualized by light microscopy. At least 200-microscopic fields from different sections of the heart and skeletal muscles tissue of mice will be screened for the presence of parasites. Comparison of the number of parasitic foci in tissue from immunized/infected mice to control infected mice will determine the effect of immunization in controlling the tissue parasite burden.

Please enter the written Sequence Listing submitted herewith.